Investigating the Mechanism of JC Polyomavirus Endocytosis

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Investigating the mechanism of JC polyomavirus endocytosis
By
Conner Robert Lajoie

A Thesis Submitted in Partial Fulfillment
of the Requirements for a Degree with Honors
(Biochemistry and Molecular & Cellular Biology)

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ABSTRACT

The majority of the human population is infected with JC polyomavirus (JCPyV), which establishes an asymptomatic infection in the kidney of healthy individuals. In immunosuppressed individuals, the virus spreads to the brain and attacks glial cells, causing progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease. With limited treatments available, an improved understanding of the virus-host cell interactions during JCPyV infection is crucial for developing effective PML therapies. JCPyV internalization into host cells requires the serotonin 5-hydroxytryptamine type 2 (5-HT2) receptors. The mechanism by which the 5-HT2Rs mediate viral entry has not yet been characterized, yet it is thought to occur by clathrin-mediated endocytosis (CME). The objective of this research is to determine whether key 5-HT2R scaffolding proteins that mediate clathrin-dependent endocytosis are required for JCPyV infection. Reduction of cellular adaptor protein 2 (AP2), adaptor protein 180 (AP180), and clathrin by siRNA significantly reduced JCPyV infection, yet inhibition of calmodulin using a chemical calmodulin inhibitor had minimal effect on JCPyV infection. Taken together, these findings suggest these specific 5-HT2R scaffolding proteins are crucial for JCPyV infection. Additionally, this research is not limited to our understanding of JCPyV viral pathogenesis, a significant human pathogen with the potential to cause fatality, but can also be applied to understanding other neurotropic viruses or other viruses that enter by CME.
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Introduction

Overview

Opportunistic pathogens have become a major concern in the realm of infectious disease. These pathogens cause asymptomatic infections in healthy individuals and are capable of causing severe and life-threatening infections in patients who are immunosuppressed (1). JC polyomavirus (JCPyV) is an opportunistic pathogen that causes progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease of the central nervous system (CNS). Specifically, JCPyV causes cytolytic destruction of oligodendrocytes, the myelin-producing cells of the CNS (2). PML has a fatality rate of 90% within the first two years of symptom onset (2). Currently, approximately 5% of individuals who are HIV-1+ are affected by PML (1), and in the past decade, the number of PML cases has risen to nearly 1:500 in patients who are receiving natalizumab, an immunomodulatory therapy designed to treat immune-mediated diseases, such as multiple sclerosis (MS) and Crohn’s disease (1,2). Thus, as the number of individuals receiving immunomodulatory therapies increases with the rise of immune-related diseases, the incidence rate of PML is anticipated to also rise. PML is a severe, debilitating disease, with affected individuals experiencing deficiencies in cognitive functioning and developing symptoms such as paralysis (1). Unfortunately, there are currently no viable treatments for this disease.

The limited treatment options for PML underscore the importance of understanding the crucial mechanisms of viral attachment and internalization that enable the virus to engage and initiate infection in target host cells. The goal of this research is to identify and characterize the role of the scaffolding proteins calmodulin, adaptor proteins 2 (AP2) and 180 (AP180), β-arrestin, and clathrin in JCPyV internalization. These studies provide insight into the mechanism of non-enveloped viral entry, which can serve
as a platform for the development of potential therapeutic targets for not only JCPyV and viral-associated PML, but also for other important human viral pathogens.

**Progressive multifocal leukoencephalopathy**

Polyomaviruses are non-enveloped double-stranded (ds) DNA viruses that infect a broad range of hosts in nature (3). JCPyV, a human-specific polyomavirus, is considered a ubiquitous pathogen because 50-60% of healthy adults are seropositive (3). There are two common strains of the virus found in humans: the archetype, non-pathogenic strain that can be isolated from the kidney and the PML-type, pathogenic strain that can be isolated from the brain of PML patients. During an asymptomatic kidney infection, the virus can undergo periods of reactivation and can be shed in the urine. Furthermore, JCPyV has been detected in untreated wastewater, suggesting that it is contracted through an oral route (1). JCPyV establishes a lifelong, persistent infection in the kidney and B cells of bone marrow (4). The majority of infected patients are asymptomatic due to an effective immune response to the infection in the kidney. However, JCPyV can undergo reactivation when seropositive adults become severely immunosuppressed and the virus spreads from sites of persistence to secondary sites of infection, including the CNS. In the CNS, JCPyV infects glial cells, astrocytes and oligodendrocytes, and causes cytolytic destruction of critical myelin-producing oligodendrocytes, which ultimately leads to PML (5).

PML is diagnosed in a clinical setting through a cranial MRI (Fig. 1) demonstrating characteristic white matter lesions in the subcortical area of the brain with no mass effect, which indicates the absence of tumors (6). The characteristic white
lesions observed on an MRI (Fig. 1) indicate areas of myelin loss. A second routine method for confirming PML diagnosis is the presence of JCPyV DNA in the cerebrospinal fluid (CSF) through polymerase chain reaction (PCR) (6). Most clinicians consider the presence of white matter lesions on the MRI and the presence of JCPyV DNA in the CSF to be sufficient to diagnose the patient with PML (6). These two methods alleviate the need for a much riskier and more invasive method of determination, such as a brain biopsy.

There are several specific populations that are at risk of developing PML (7), with the two largest being those infected with the human immunodeficiency virus (HIV-1+) and those with multiple sclerosis (MS) who are taking natalizumab as an immunosuppressive treatment (7). Additionally, there are distinctions between these populations regarding how individuals become immunosuppressed (i.e., a virus or an immunomodulatory therapy). Patients who are immunosuppressed by HIV-1 infection tend to have PML symptoms associated with weakness, speech abnormalities, and gait abnormalities (8). By contrast, those who have natalizumab-induced PML tend to suffer from cognitive disorders, motor abnormalities, and visual defects (8). It has been suggested that this could be due to the fact that the lesions of natalizumab-induced PML patients are primarily monofocal and develop in the frontal lobe (8). In comparison, HIV-
1 induced PML lesions tend to be multifocal, meaning they are in multiple lobes of the brain (8).

The most at-risk population is the HIV-1+ population, which constitutes approximately 80% of annual PML cases (7). Unfortunately, there are no effective clinical treatments for PML. The only partially effective course of action is immune constitution. In HIV-1+ patients, the immune system is deficient and the pathogenic strain of JCPyV is capable of spreading to the brain (7). Currently, the common treatment for HIV-1 is highly active antiretroviral therapy (HAART). HAART is a combination of anti-viral drugs that treat the HIV-1 infection and restore the immune system function in patients who are immunosuppressed. Before the development of HAART, PML occurred in 3-7% of all HIV-1 cases, accounted for 18% of all fatal HIV-related CNS infections, and had a 95% mortality rate (9, 10). Since the development of HAART, the mortality rate has fallen from 95% to 30% within one year of symptom onset and down to 60% within two years of symptom onset in HIV-1+ patients (7). The incidence of PML has also substantially decreased since the introduction of HAART. In a yearly representation, the incidence of PML in HIV-1+ patients has decreased over time, from 14.8 cases per 1,000 in 1996 to 2.6 cases per 1,000 in 2005, and further, to 0.8 cases per 1,000 in 2011 (11).

The second largest population at-risk for PML are patients who are receiving immunomodulatory therapies for immune-related diseases. The highest incidence of PML in this population is among individuals with MS under treatment with the drug natalizumab (11). This drug is a humanized monoclonal antibody that blocks autoreactive T cells from trafficking to the brain and attacking myelin, a process that constitutes the
underlying etiology of MS (7). However, the lack of T cells trafficking to the brain results in decreased immune surveillance and is suggested to result in JCPyV reactivation in the kidney and spread to the CNS, causing PML (8,9,11). Since 2005, there have been over 711 reported cases of PML development in patients receiving natalizumab treatment (7). For patients who developed PML from natalizumab treatment, the recommended treatment is to stop taking the medication in an effort to reconstitute the immune system. However, removal of the therapy can cause PML-immune reconstitution inflammatory syndrome (PML-IRIS), an overwhelming inflammatory response that can result in neurological worsening, long-term debilitation, and is often in itself, fatal (11).

Immunomodulatory drugs with life-threatening risks like PML are required by the FDA to include a “black box warning” in the prescribing information (12). Natalizumab has a black box warning but is regularly prescribed for MS. Even new drugs that have recently come to market that have had no incidence of PML development during the extensive FDA trial programs, still list PML in a black box warning to inform the consumer that there is a chance of life-threatening side effects.

**JCPyV attachment and entry**

The first step in the infectious life cycle of viruses is attachment to cell surface receptors and subsequent entry into host cells (13). The majority of polyomaviruses studied to date use a sialic acid receptor to bind to the surface of target cells (13). JCPyV binds to host cells via interactions of the outer capsid protein, viral protein 1 (VP1) with the α2,6-linked glycan, lactoseries tetrasaccharide c (LSTc) receptor motif (13). There is some evidence that JCPyV uses a clathrin-mediated endocytotic pathway that involves cell
surface serotonin hydroxytryptamine (5-HT)\textsubscript{2} family of receptors (14,25,27). Following endocytosis presumably into a clathrin-coated pit, then trafficking to an endosome, the enclosed vesicle traffics through the endocytic compartment to the endoplasmic reticulum where partial uncoating occurs through the endoplasmic-reticulum-associated protein degradation pathway (ERAD) (15). The partially uncoated virion subsequently traffics to the nucleus where the virus is completely uncoated and the double stranded DNA genome can be transcribed and replicated. JCPyV has temporal gene regulation; expression of early gene T-antigen (T-Ag) drives genome replication and the production of the late capsid structural viral proteins 1, 2, and 3 (VP1, VP2, VP3) (15). Following replication, the capsid is assembled around newly replicated genomes and virions are packaged and released from the cell.

All subtypes of the 5-HT\textsubscript{2} serotonin receptor family; 5-HT\textsubscript{2A}, 5-HT\textsubscript{2B}, and 5-HT\textsubscript{2C}, rescue JCPyV infection in non-permissive cells and enhance entry into cells, but the mechanism by which this occurs remains unclear (4, 17). Previous studies have suggested that JCPyV does not interact with the extracellular loops of 5-HT\textsubscript{2}Rs on the cell surface ((13,14) and Atwood Lab, unpublished results)). Thus, the 5-HT\textsubscript{2} serotonin receptors play
a critical role in JCPyV entry, yet the specific mechanism by which these receptors facilitate viral entry remains unclear.

**Serotonin receptors**

Serotonin receptors of the 5-HT\textsubscript{2} subfamily are important neurotransmitter receptors in the brain, nervous system, and peripheral tissues (16). The 5-HT\textsubscript{2} receptors are seven transmembrane-spanning G protein coupled receptors (GPCRs) with three extracellular loops and three intracellular loops.

The intracellular loops contain specific binding domains for G proteins and other scaffolding proteins that when bound activate cellular endocytosis and signaling (16). After activation by ligands, 5-HT\textsubscript{2}Rs can activate two distinct signaling pathways, a G-protein coupled mechanism, and a \(\beta\)-arrestin mediated signaling pathway, both of which converge on the activation of the mitogen-activate protein kinase pathway (MAPK) (Fig. 3). Additionally, desensitization of the receptor and subsequent G-protein uncoupling also activates calmodulin; the reaction cascade causes a calcium flux from the ER and further binding and stabilization of

![Figure 3: 5-HT\textsubscript{2}Rs activation and signaling](image)

Following the activation of the receptor, classical GPCR signaling is associated with calmodulin recruitment. \(\beta\)-arrestin recruitment causes uncoupling of the G protein through desensitization of the receptor. The uncoupling event leads to clathrin-mediated endocytosis. Clathrin-mediated endocytosis requires the scaffolding proteins clathrin, AP2, AP180, dynamin, and \(\beta\)-arrestin. This internalization is associated with calmodulin-receptor signaling cascades that promote the MAPK-ERK cascade, which results in an increase in activation and nuclear transcription factors (19). Image courtesy of Dr. Melissa Maginnis.
calmodulin to the serotonin receptor (18). This activation allows calmodulin to also activate the MAPK pathway to stimulate downstream signaling (16) with the MAPK pathway acting as a kinase cascade that results in the activation of extracellular signal regulated-kinases (ERK), a transcription factor known to be crucial for JCPyV infection (1). 5-HT2 receptor antagonists are utilized in the treatment of neurological disorders, including mood disorders like depression, (18) and thus serve as a potential target as a JCPyV and PML therapeutic. However, it is essential to first understand the exact mechanism by which JCPyV is utilizing the 5-HT2Rs to determine which drugs can be put forward in testing based on their exact target.

Previous research has demonstrated that the 5-HT2A, 5-HT2B, and 5-HT2C receptors are crucial to JCPyV infection (4). These receptors only have a 50% sequence identity, but have key intracellular motifs that are conserved throughout (4). The 5-HT2AR contains two calmodulin (CaM)-binding domains, referred to herein as domain 1 and domain 2. CaM is a ubiquitous calcium (Ca\(^{2+}\)) binding protein sensor, as it is activated and regulated by Ca\(^{2+}\) binding. CaM has four Ca\(^{2+}\) binding sites. Ca\(^{2+}\) binding causes a conformational change that activates CaM to serve as a secondary messenger. Once activated, CaM can bind to specific sites on the serotonin 5-HT2 receptors (14). The 5-HT2A receptor contains four intracellular motifs that are important to clathrin-mediated endocytosis. The (Ala-Ser-Lys) ASK and (Asn-Pro-any amino acid (X)-X-Try) NPXXY domains serve as two potential binding sites for the
CME associated protein, β-arrestin (17). The two CaM-binding sites in the intracellular loops of the receptor consist of a 1-8-14 motif (domain 1) and a 1-10 motif (domain 2) with hydrophobic residues and potential CaM binding sites at positions 1, 8, 10, and 14 (14) (Fig. 4). These motifs undergo a conformational change when bound by CaM that stabilizes the receptor on the cell surface to allow for interaction with ligands via receptor-mediated endocytosis, thus activating signaling events (16,17,18). Therefore, it is expected that the presence of serotonin receptors on the surface of the cell is key to the internalization and subsequent infection by JCPyV.

The β-arrestin ligand signaling pathway is activated by viral attachment to the cell and causes the uncoupling of the G-protein from the serotonin receptor (17). The uncoupling event is thought to be facilitated by the binding of calmodulin and the activation of β-arrestin (19). The activation of β-arrestin facilitates the internalization of the 5-HT2A receptor via clathrin-mediated endocytosis. Clathrin-mediated endocytosis requires the recruitment of endocytic scaffolding proteins, such as AP2 and clathrin. In turn, these proteins stimulate downstream signaling cascades that promote the activation of cellular transcription (Fig. 2). Preliminary data from the Maginnis Laboratory has shown that β-arrestin and the MAPK pathway are crucial for JCPyV infection. Understanding how JCPyV is able to tip the activation of the serotonin receptor away from the G-protein coupled signaling and towards the β-arrestin mechanism is essential to understanding how drugs could be used to target 5-HT2Rs signaling pathways and serve as effective therapeutics.

In an attempt to treat PML, clinicians have prescribed chemical antagonists that target the reuptake of serotonin, such as selective serotonin reuptake inhibitors (SSRIs) in
off-label treatment regimens. Several studies have assessed the use of different drugs that target serotonin receptors in open label or anecdotal PML studies. One serotonin antagonist that has been tested is the antidepressant mirtazapine. Mirtazapine has been shown to selectively inhibit 5-HT$_2$ and 5-HT$_3$ receptors (20) and block JCPyV infection in human glial cells (21). Reports of these anecdotal trials demonstrate that mirtazapine treatment has varying degrees of efficacy in PML patients. There is evidence that mirtazapine can improve the prognosis of patients who are diagnosed with PML. Indeed, many studies have shown a significant decrease in the neurological deterioration and detectable viral loads in the blood and CSF (22). On the other hand, reports have yielded results that suggest mirtazapine is largely ineffective. In these studies, patients’ conditions rapidly deteriorated despite being treated with the 5-HT$_3$R antagonist, and there were no remarkable MRI changes in the infected patients (23). Thus, it is questionable whether or not mirtazapine alone is an effective treatment of PML, but studies have shown that it can be more effective when used with an anti-parasitic drug, mefloquine, which has been shown to decrease JCPyV replication in vitro (7). Mefloquine, a treatment for malaria, has been shown to be effective in treating individuals with PML by causing a decreased JCPyV infection after the virus has entered the cell (24,25,26). Taken together, these results suggest that mirtazapine may be efficacious in preventing cell-to-cell spread of JCPyV by blocking infection of new cells while mefloquine may be capable of treating underlying JCPyV infection where infection has already been established. While the reports for use of mirtazapine and mefloquine are promising, the conflicting evidence between these reports suggests that further investigation and exploration is needed in determining the method of JCPyV-mediated
infection by 5-HT₂Rs. There should also be continued exploration of off-label use of approved therapies in the treatment of JCPyV and PML.

**Clathrin-mediated endocytosis**

JCPyV is a non-enveloped virus; it must traverse the host cell membrane in order to establish infection and is unable to fuse with the membrane like enveloped viruses (27). Therefore, the virus must be internalized via a cellular endocytic pathway such as clathrin-mediated endocytosis, micropinocytosis, or caveolin-dependent endocytosis (27). It is proposed that JCPyV uses clathrin-mediated endocytosis for viral entry because chemical inhibitors of clathrin-mediated endocytosis, including chlorpromazine, limit JC virus infection (27). However, the mechanism by which this occurs is not well understood because chlorpromazine has been shown to act as an antagonist of serotonin receptors (25). CME is a process that is inherent to all cells as a method of internalizing host factors that are needed for homeostasis and proliferation. Further, it is unknown whether JCPyV-mediated CME occurs through direct virus-host interactions with the 5-HT₂ receptors. Previous literature has shown that JCPyV infection of host glial cells requires the internalization of the CME associated protein, epidermal growth factor 15, eps15 (27). There is additional evidence that that dominant-negative eps15 mutants inhibit clathrin-dependent endocytosis (27). The dominant negative mutants also caused a decrease in JCPyV internalization, further demonstrating that JCPyV likely enters the cell in a clathrin-mediated fashion. This method of entry is different than its most closely related polyomavirus, SV40. Even though they are very similar, SV40 uses sialic-acid-containing ganglioside receptors and enters via a non-clathrin mediated mechanism (27).
Clathrin-mediated endocytosis is a complex and diverse cellular entry pathway and has been chiefly reported to require cellular scaffolding proteins AP2, AP180, and clathrin. However, activation of proteins involved for internalization is ligand-specific and may vary based on the receptor utilized in addition to receptor-ligand activation. Both AP2 and AP180 are able to bind to β-arrestin and recruit clathrin to the cell surface (26). The classical view is that CME is initiated by the recruitment of AP2 to the plasma membrane of the cell (28) and acts in conjunction with AP180 to attract clathrin for the invagination of the receptor and virus (Fig. 5). This view is supported by research that found that the mutation of AP2 binding sites in the eps15 protein significantly decreased the infection of a virus that uses CME (29). It has been shown that the use of siRNA against the µ2 subunit of AP2 showed a significant decrease in the formation of clathrin coated pits and vesicles, as the µ2 subunit contains a conserved motif capable of interacting with GPCRs and facilitating CME pit formation (28). This indicates that AP2 is an important scaffolding protein capable of interacting with β-arrestin and can limit CME, but it is not the only scaffolding protein that is crucial for CME.

AP180 is thought to act in a combinatorial manner with AP2 in clathrin recruitment to the membrane of cells, yet the mechanism is not well understood (30).

**Figure 5: Clathrin-mediated endocytosis.** Ligand binding to receptors leads to recruitment of AP2 and clathrin to the plasma membrane (Binding/Recruitment). Membrane curvature forms a pit inside the cell (Invagination/Maturation). Scission by dynamin causes internalization of cargo in a clathrin-coated vesicle (36) Used by permission from MBInfo.
Studies have demonstrated that AP2 and AP180 colocalize and potentiate the binding of clathrin to clathrin-coated vesicles (30). There is additional evidence that AP180 is four times more efficient and effective at recruiting and binding to clathrin (28). Even though AP180 is four times more effective at binding to clathrin, it has been shown that AP2 and AP180 work together and are most effective when colocalized on the clathrin-coated vesicle (30). Similar to AP2, the reduction of one protein does not completely abolish the presence of clathrin-coated vesicles, likely indicating that the APs are capable of recruiting clathrin in the absence of the other.

The most important protein for CME is clathrin itself. Upon recruitment of AP2 and AP180 to the plasma membrane, clathrin is recruited to the membrane to serve as an outside coat for the vesicle (29). Interestingly, clathrin does not directly interact with the invaginating membrane or the activated receptor, but has direct interactions with scaffolding machinery around the pit. Once the vesicle is internalized into the cell, the clathrin coat detaches and the contents of the vesicle become accessible to the cellular endocytic pathway. It has been shown that the abolishment of the heavy chain of clathrin causes a significant reduction in the presence of clathrin-coated vesicles in cells (30). This finding demonstrates that clathrin is crucial for clathrin-mediated endocytosis. If JCPyV requires clathrin-mediated endocytosis for infectious entry, then the abolishment of any of these proteins in vitro will likely impact infection.

**Research goals**

The research discussed in this thesis explores the role of CME-scaffolding proteins and 5-HT$_2$R-protein interaction domains in JCPyV infection of human glial cells, resulting in
JCPyV entry limitation by the abolishment of the clathrin-mediated scaffolding proteins AP2, AP180, and β-arrestin, but not by the limitation of calmodulin. Entry was further prevented by degrading clathrin, the key protein involved clathrin-mediated endocytosis. These studies were conducted to further build on published literature and unpublished work in the Maginnis Laboratory, which demonstrate that JCPyV is internalized into its human host cells through clathrin-mediated endocytosis. This information will contribute to the broader understanding of non-enveloped viral entry through clathrin-mediated endocytosis. In addition, this research will provide new information about JCPyV entry and possible mechanisms that can be targeted in the development of new antiviral therapies.

**Materials and Methods**

**Cell culture maintenance**

A Lab Gard II laminar flow hood was used to limit contamination while conducting cell culture experiments. SVG-A and HEK293A (Human Embryonic Kidney) cell lines were used and cultured in T75 flasks that were grown in a humidified incubator at 37°C with 5% CO₂. Both lines were passaged upon viewing approximately 90% confluence under an inverted microscope. The SVG-A cell line was cultured in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S) (Mediatech, Inc.), and 0.2% plasmocin (Invivogen). HEK293A cells were cultured in similar conditions, except Dulbecco’s Modified Eagle Medium (DMEM) were used in place of MEM. Both lines are adherent and were detached by a 5-minute incubation at 37°C with 4ML of 0.5% trypsin-EDTA (Gibco by
Thermo Fischer Scientific). Following incubation, 9mL of fresh media was added to the flask. The media, now containing the detached cell line was transferred to a 15mL conical tube. The tube was centrifuged at 2,000 rpm for 5 mins. Pelleted cells were resuspended in fresh complete growth media and divided to a new flask based on the desired growth period. The new flask was then cultured in a humidified incubator at 37ºC and 5% humidity until reaching confluence; at that time the process was repeated.

**JCPyV and SV40 infection**

Cells were treated with a chemical inhibitor or transfected with siRNA, before media was removed and the cells were infected with JCPyV or SV40 (MOIs indicated in Figure Legends). Viral infection was conducted in MEM with 2% FBS and no antibiotics in a 200µL volume. After a 1-hour incubation at 37ºC, cells were fed with 1mL of complete MEM and allowed to incubate at 37ºC for 72 hrs before being fixed and stained by indirect immunofluorescence.

**Calmodulin inhibition**

Calmidazolium chloride (Santa Cruz Biotechnology) was reconstituted in DMSO and used at indicated concentrations. SVG-A cells were plated at $2.5 \times 10^4$ cells per well in a 24 well plate overnight. Upon 70% confluency, cells were treated with indicated concentrations of inhibitor for 3 hrs prior to infection. Cells were immediately infected after the pre-treatment with JCPyV (MOI of 1 FFU/cell) in MEM containing 2% FBS and incubated at 37ºC for 1 hour. After the incubation, cells were fed with 1mL of room temperature MEM with 10% FBS and 1% P/S, incubated for 72 hrs at 37ºC, then fixed
and stained by indirect immunofluorescence. Infection was quantified by the number of VP1 positive nuclei in a visual field.

**Indirect immunofluorescence**

Following infection, cells were washed once with 1xPBS then fixed in ice cold methanol. Fixed cells were incubated at -20°C for greater than 10 mins before staining. After incubation at -20°C, cells were washed with 1xPBS 3 times for 10 mins each followed by a 15-minute incubation with PBS-0.5% TX100 for permeabilization of the cellular membranes. Cells were stained with a primary antibody, PAB597 (1:10) a hybridoma supernatant that produces a monoclonal antibody against JCPyV VP1 (33) and was generously provided by Ed Harlow. A secondary anti-mouse Alexa Fluor 488 antibody (Thermo Fisher Scientific) was used to detect VP1+ cells. Cells were quantified for infectivity by fluorescence microscopy by counting VP1+ cells in at least 5 visual fields per well in triplicate wells for 3 separate experiments. Expression of VP1 was observed under a 10x objective using a Nikon Eclipse Ti epifluorescence microscope. Percent infection was quantified by the number of VP1+ nuclei per visual field divided by the number of DAPI positive nuclei in the same field. The number of DAPI positive cells were quantified using a binary created in the Nikon NIS-Elements Basic Research software (Version 4.5), controlling for equal diameter and circularity of DAPI-stained nuclei based upon threshold fluorescence. The average percentage of infected cells was then normalized to the EGFR control siRNA. Significance was determined using a Students’ t-test (Microsoft Excel).
Flow cytometry to measure serotonin receptor expression

HEK293A cells were transfected with mutated 5-HT$_{2A}$ serotonin receptors in a 6 well plate (0.5e$^6$ cells per well) and allowed to grow overnight to 100% confluence. Media was aspirated from the wells and all of the wells were washed with 1xPBS. The cells were then incubated in Cellstripper (Corning). Cells were pooled in 1.5mL microcentrifuge tubes, pelleted at 2500 rpm for 5 mins at 4ºC, and washed with PBS. Cells were incubated with a 1:250 dilution of the 5-HT$_{2A}$ receptor primary antibody, SR-2A (Santa Cruz Biotechnology), for 30 mins at 4ºC. After the incubation, the cells were pelleted at 2500 rpm for 5 mins and washed 3 times with PBS. A total of 100µL of the cells were transferred to a new tube and were incubated with the secondary antibody, anti-rabbit Alex Fluor 488 antibody (Thermo Fisher Scientific). Incubation was conducted on ice for 2 hrs with agitation of the solution every 15 mins. Cells were washed, pelleted by centrifugation at 2500 rpm, and resuspended in 500µL of PBS. Cells were analyzed for 5-HT$_{2A}$ receptor expression of the cell surface using a BD FACSCanto (BD Biosciences) equipped with a 488 laser excitation line (Benton, Dickinson, and Company). The data generated by this experiment were analyzed using BD FacsDIVA (Benton, Dickinson, and Company) and FlowJo software (Tree Star, Inc.).

Mutation of serotonin receptors

Calmodulin binding domains in the 5-HT$_{2A}$ receptor were mutated using a Qiagen site-directed mutagenesis kit. All of the sites (F186, V193, F199, L382, and F391) were mutated to alanine residues. Mutagenesis was previously performed by Dr. Melissa
Maginnis. Following the transformation, plasmids were amplified and isolated using a Qiagen Maxi Prep kit.

**Transfection of mutated receptors**

HEK293A cells were plated to 80% confluence in a 24 well plate at the time of transfection. Before addition to the wells, 0.5µg/well of the receptor plasmids were mixed in incomplete medium lacking serum or antibiotics. In a separate tube, 1µL/well of Lipofectamine 2000 (L2000) (Thermo Fisher Scientific) was mixed with incomplete medium lacking serum or antibiotics (Invitrogen). Plasmids and L2000 were incubated in their respective tubes for 5 mins then before combined. Combined samples were mixed well and incubated at room temperature for 15 mins. After incubation, 100µL were added per well in a dropwise fashion and allowed to incubate in a humidified, 37ºC incubator for 4 hrs. Following the incubation, the media was removed and 1mL of complete media containing serum and antibiotics was added to cells. Transfection of plasmids was allowed to progress for 24 hours before experimental use.

**siRNA treatment**

SVG-A cells were plated to approximately 50% confluence in 10% FBS MEM without antibiotics in a 12 well plate. Cells were then allowed to adhere for 4 hrs before transfection with siRNAs specific for AP2 (Dharmacon), β-arrestin (Cell Signaling Technology), AP180 (ThermoFischer), clathrin heavy chain (Life technologies), unconjugated non targeting control (Cell signaling technology 65685), or an EGF receptor control (Cell Signaling Technology 64825). β-arrestin, clathrin heavy chain,
AP180, and AP2 siRNA (30 or 60 pmol) was complexed with RNAiMax transfection reagent (Invitrogen) in serum- and antibiotic-free MEM (Corning) and incubated at room temperature (RT) for 10 mins. Transfection mixtures were added to cells incubated in 1mL of serum- and antibiotic-free MEM dropwise. Transfections were performed as follows: β-arrestin siRNA: 30pmol for 72hrs; clathrin heavy chain siRNA: 30pmol for 24hrs, then washed in MEM and transfected with 30pmol for 48hrs; AP2 and AP180 siRNA (single or double transfections): 30pmol for 72hrs or 30pmol for 48hrs, then washed in MEM and transfected with 30pmol for 24hrs for double transfection samples. All siRNA transfections were allowed to proceed for 72hrs total prior to infection.

Transfection efficiency was monitored using Block-iT Alexa Fluor Red Control Oligo (Life Technologies) visualized at 48 hrs post transfection by fluorescence microscopy. Complexes were added to each well in 100μL volumes in a dropwise fashion and incubated at 37°C for 72 hrs; the transfection medium was not removed. At 72 hrs post-transfection, cells were infected with JCPyV or SV40 in MEM with 2% FBS and incubated at 37°C for 1 hour. Cells were fed with 1mL of complete MEM containing antibiotics, incubated for 72 hrs, then fixed with 100% ice cold methanol and stained by indirect immunofluorescence. The percentage of infected cells was quantified as described previously.

**siRNA western blot analysis**

In order to analyze protein knockdown following siRNA treatment, SVG-A cells were washed with PBS after a 72-hour treatment of the AP2 siRNA. Cells were scraped from the wells and pelleted by centrifugation at 2500 rpm at 4°C for 5min. Pellets were
resuspended in 50µL of Tris-HCl lysis buffer that contained both protease (1:10) and phosphate (1:100) inhibitors. Samples were incubated on ice for 10 mins before centrifugation at 18,600 rpm at 4°C for 10 mins. The supernatants were transferred to a new tube and were mixed (1:1) with 2X Laemmli sample buffer containing β-mercaptoethanol. Samples were resolved on a 10% SDS-PAGE TGX gel (BioRad) that was run at 9 milliamps in a Bio-rad Mini gel box. Proteins were transferred to a nitrocellulose membrane using the rapid blot transfer program on the Trans-Blot Turbo Transfer System (BioRad). Membranes were blocked using 5% non-fat dry milk with 0.1% TBS-T overnight. Blots were then washed three times at RT for 10 mins with TBS-T (0.1% Tween) while vigorously rocking. Following washing, the blot was incubated overnight while rocking at 4°C with an antibody specific for AP2 (1:1000) (Cell Signaling Technology) and polyclonal antibody for GAPDH (loading control) (1:2000) (Abcam) in 5% BSA/ 0.1% TBS-T. Membranes were washed in TBS-T at RT, then incubated with a secondary antibody at 1:15,000 (LICOR) dilution at RT for 1 hour in 5% milk with TBS-T. Membranes were washed in TBS-T and TBS, then imaged using a LICOR Odyssey Clx. Results were quantified using the Image Studio program (LICOR).

Results

Clathrin inhibition decreases JCPyV infection

Previous studies have shown that chemical inhibition of the clathrin-mediated endocytotic pathway reduces JCPyV infection (4,34). To further determine whether clathrin-mediated endocytosis is crucial to infection, key proteins, including clathrin, were targeted through siRNA treatment. SVG-A cells were transfected with a siRNA
targeted toward clathrin heavy chain to reduce clathrin protein levels in cells (Fig. 6). Cells were then infected with JCPyV and infection was quantitated by indirect immunofluorescence in a viral fluorescent focus unit (FFU) assay.

Cells expressing a clathrin siRNA demonstrated an approximately 80% decrease in infection in comparison to cells treated with a control siRNA (Fig. 6). These data suggest that clathrin is crucial for JCPyV infection.

**The role of calmodulin in JCPyV infection is inconclusive**

We hypothesized that the interaction of calmodulin with the 5-HT$_2$R plays a crucial role in JCPyV infection. To investigate the role of calmodulin in JCPyV infection, SVG-A cells were treated with a calmodulin antagonist, calmidazolium chloride, infected with JCPyV, and...
infection was scored using an FFU assay. Cells treated with calmidazolium chloride led to a significant decrease in infection at concentrations at or above 0.75-1µM (Fig. 7). However, treating cells with concentrations greater than 1µM of calmidazolium chloride did not result in a dose-dependent decrease in infection. This suggests that the antagonist may have a secondary effect that is limiting infection, or the maximal efficacious inhibitor concentration was reached without compromising cellular viability. An MTS assay was performed to view cellular viability. There was a significant decrease in cellular proliferation between a 0.75µM and 1µM treatment of calmidazolium chloride (data not shown).

To further investigate the role of calmodulin, and to define direct calmodulin-receptor interactions, serotonin receptors with specific calmodulin binding domain mutations were generated. Each of the key amino acids in the 1-8-14 and 1-10 motif of

![Figure 8: 5-HT2A receptor cell surface expression.](image)

<table>
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<th>Sample Name</th>
<th>Median: HT2A</th>
</tr>
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</tr>
<tr>
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</tr>
<tr>
<td>5HT2AR-F391A</td>
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**Figure 8: 5-HT2A receptor cell surface expression.** HEK293A cells were transfected with the transfection reagent (L2000) and the wild-type and mutant 5-HT2ARs for 24 hours. After transfection, the cells were stained with a 5-HT2AR specific antibody and complementary secondary fluorescent antibody. Fluorescence was quantified by flow cytometry. Histograms represent the median fluorescence intensity for 10,000 events.
the 5-HT$_{2A}$ receptor, F186, F199, V193, L382, and F391, were mutated to alanine. The serotonin receptors with mutated CaM-binding domains were transfected into HEK293A, a poorly permissive cell line. Previous research by the Maginnis Lab has shown that when the mutated receptors were transfected into HEK293A cells then infected with JCPyV there was a significant decrease in infection in the F391A mutant receptor yet no difference in infection was observed for other mutations tested (Maginnis Lab, unpublished data). To determine if this decrease was due to a lack of calmodulin binding or a decrease in the ability of the receptor to be expressed on the cell surface, the cell surface expression of the mutated receptors was analyzed. HEK293A cells were transfected with the wild-type and mutant 5-HT$_{2A}$ receptors and stained with a serotonin-specific antibody and complementary secondary fluorescent antibody, and cell surface expression of the receptor was determined by flow cytometry. All of the mutated receptors were expressed on the cell surface at levels greater than the vehicle control (L2000) but at levels that were lower than the wild type 5-HT$_{2A}$ receptor (Fig. 8). The mutated receptor had reduced cell-surface expression in comparison to the wild-type 5-HT$_{2A}$ receptor, and (F391A) was the only receptor that had a decreased infection (Fig. 8. and Maginnis Lab, unpublished data). Thus, the decrease in infection in cells expressing the F391A mutant could be due to reduced surface expression of the receptor. Taken together, these data are not sufficient to determine whether calmodulin is crucial to JCPyV infection, and the role of other scaffolding proteins involved in CME were further explored.
siRNA targeting of β-arrestin, AP2, and AP180 significantly decreases JCPyV infection

β-arrestin binds to the 5-HT₂R β-arrestin domain, then scaffolds and recruits AP2, AP180, and clathrin (18). It also plays an important role in the uncoupling of G proteins from serotonin receptors (18). This data suggests that β-arrestin may play a crucial role in JCPyV entry in a clathrin-mediated endocytic manner. To explore this further, SVG-A cells were transfected with a siRNA specific to β-arrestin and infected with JCPyV or SV40 polyomavirus as a control (Fig. 9). Cells treated with the β-arrestin siRNA demonstrated an 80% decrease in JCPyV infection compared to cells expressing a control siRNA, while no significant reduction was noted in the SV40 infection (Fig. 9). These data suggest that β-arrestin is crucial to JCPyV infection and that JCPyV enters host cells in a clathrin-mediated pathway that is distinct from the SV40 endocytotic pathway.

To further demonstrate that JCPyV uses clathrin-mediated endocytosis, an AP2-specific siRNA was used to reduce AP2 expression. Cells were transfected with the AP2 siRNA, followed by infection with JCPyV or SV40 (Fig. 10A), resulting in a ~70% decrease in JCPyV infection in cells treated with an AP2 siRNA compared to cells treated...
with a control siRNA (Fig. 10A). Comparatively, SV40 infection was not impacted in cells that were treated with an AP2-specific siRNA or a control siRNA. These data suggest that AP2 is also crucial for JCPyV infection.

To confirm that the AP2 siRNA was specifically reducing the levels of AP2 protein in the cell, a western blot was performed (Fig. 10B). SVG-A cells were treated with an AP2-specific siRNA or a control siRNA and cell lysates were resolved by SDS-

![Figure 10](image-url)

**Figure 10: AP2 degraded by siRNA significantly impacts JCPyV infection.** (A) SVG-A cells were pretreated with a siRNA oligo specific to AP2 or control (EGFR). The cells were then incubated for 72 hrs. Following this incubation, cells were infected with either JCPyV or SV40 (MOI of 0.1 and .0001 FFU/cell respectively) and infection was allowed to progress for 72 hrs. Data represent the average percentage of infected cells per visual field for five visual fields for AP2 siRNA in comparison to a siRNA control. (B) SVG-A cells were pretreated with an AP2-specific siRNA for 72hrs in a 6 well plate. Following incubation, proteins were run on a SDS page gel and stained with a secondary antibody for AP2 or GAPDH (control). *, P <0.01 denotes significance determined by students’ t test.

PAGE and analyzed by western blot using an antibody specific to AP2 or a control GAPDH and complementary secondary antibodies. Protein levels were quantified using a LiCOR Odyssey Clx, and there was a 75% decrease in AP2 protein in the cells treated with the AP2 siRNA compared to cells treated with the control siRNA (Fig. 10B). This data suggests that the siRNA for AP2 is working properly and degrading the AP2 protein.
To explore the impact of AP180 in an absence or presence of AP2 in JCPyV infection, cells were transfected with an AP2 siRNA, AP180 siRNA, or a mixture of both siRNAs (Fig. 11). It was observed that the AP180 siRNA had a 60% decrease in infection and the AP2 siRNA had a 35% decrease in infection when compared to a control siRNA (Fig. 11). The dual siRNA treatment had the most significant effect on infection and decreased infection by 80% when compared to a control siRNA. These results indicate that while both adaptor proteins are required for infection, the impact of AP180 is greater than that of AP2. Additionally, this further suggests that AP2 and AP180 are crucial to JCPyV infection and that AP2 and AP180 may act in a collaborative fashion because an additive effect was observed. These data suggest that JCPyV requires AP2 and AP180 for successful infection of human glial cells.

**Discussion**

JCPyV was previously shown to require the activity of 5-HT₂R serotonin receptors for viral entry into host cells (4). Furthermore, evidence has demonstrated that JCPyV entry occurs in a clathrin-dependent manner (4). However, the evidence that led
to the conclusion that JCPyV enters cells by clathrin-mediated endocytosis is based on the finding that chlorpromazine can inhibit JCPyV infection (23). While chlorpromazine is widely used in the field of virology to limit clathrin-mediated endocytosis, it is also a 5-HT2R serotonin receptor antagonist (23) and is thus not an appropriate inhibitor for use in JCPyV studies. Therefore, the mechanism of JCPyV entry required further investigation using more specific inhibitors and additional methods to determine whether clathrin and related scaffolding proteins are essential for JCPyV entry into host cells. Herein, I have demonstrated that JCPyV infection is dependent upon clathrin, as evidenced by a significant reduction in JCPyV infection in cells treated with an siRNA directed toward clathrin heavy chain (Fig. 6). Though calmodulin is thought to play a role in uncoupling the classical GPCR G-protein signaling pathway and activating a β-arrestin specific endocytic pathway, inhibition of calmodulin by a chemical inhibitor did not have a significant effect on JCPyV infection (Fig. 7), indicating that calmodulin may not be involved in JCPyV infection and requires further exploration. However, gene silencing of β-arrestin, a scaffolding protein that can bind directly to 5-HT2Rs, and other scaffolding proteins essential for clathrin-mediated endocytosis including AP2 and AP180, significantly prevented viral infection (Fig. 9, 10, and 11), indicating that JCPyV utilizes clathrin-mediated endocytosis to infect host cells.

Despite previous evidence that calmodulin plays a role in JCPyV infection (Maginnis Lab, unpublished results), we found conflicting results that render our data inconclusive. We investigated the role of calmodulin in JCPyV infection as it has been previously reported that calmodulin binding to the serotonin receptor activates the receptor and plays a role in endocytosis and activation of signaling events (18,19).
However, treatment of cells with the inhibitor calmidazolium chloride led to a decrease in JCPyV infection, yet the reduction in infection was not dose-dependent (Fig. 7). An MTS toxicity assay revealed that calmidazolium chloride was toxic to the cells at higher concentrations (data not shown). Therefore, the chosen inhibitor was not acting as expected and was most likely having secondary effects, or high enough concentrations necessary to inhibit calmodulin were not obtainable due to cellular toxicity effects.

Further, we demonstrated that a decrease in infection could be achieved by mutating the phenylalanine at position 391 to an alanine (F391A), but this receptor was shown to have reduced cell surface expression by flow cytometry compared to the wild type 5-HT$_2$A receptor (Fig. 8). This indicates that the decrease in infection demonstrated by the F391A mutant could be due to a loss of calmodulin interaction or that there are fewer receptors on the surface that could be utilized for entry. Taken together, these data are not conclusive to determine the role of calmodulin in JCPyV entry. To further define the role of calmodulin in JCPyV infection, an additional chemical inhibitor that limits calmodulin in the cell should be explored. Additionally, further research should be conducted into the residues that interact with calmodulin by making multiple mutations, instead of single point mutations in 5-HT$_2$A.

We also demonstrated that JCPyV uses a clathrin mediated-endocytic entry pathway as gene silencing of key scaffolding proteins involved in 5-HT$_2$R internalization, $\beta$-arrestin, AP2, AP180, and clathrin, significantly reduced infection. Interestingly, cells that received a double AP2 siRNA treatment (Fig. 10A) had an even greater decrease in JCPyV infection and cellular toxicity than those that received a single treatment of AP2 or AP180 siRNA (Fig. 11). This observed effect could be due to the fact that a double
transfection of siRNA against the AP2 µ2 subunit causes the knockdown of the alpha and beta appendages that interact with AP180, which has been previously demonstrated in the literature (37). This indicates that the significant decrease in a dual siRNA treatment of AP2 could be due to the limitation of both AP2 and AP180 rather than just AP2. Taken together, these data suggest that JCPyV is utilizing a mechanism for entry similar to that of the activated 5-HT₂R receptor.

The closely related polyomavirus, SV40, was used as a control, and treatment of cells with siRNAs specific for β-arrestin, AP2, AP180, and clathrin, had no effect on SV40 infection (Fig. 9 and 10. and not shown data.) This confirms that the activation of clathrin-mediated endocytosis in SVG-A cells is specific for JCPyV as it differs from a close viral relative. This data further aids our understanding of the processes involved in JCPyV entry and, more broadly, how viruses are able to enter cells. Future studies will focus on defining the role of other 5-HT₂R clathrin mediated endocytic proteins in JCPyV infection and exploring whether inhibition of these scaffolding proteins plays roles in the ability of the virus to bind to host cells.

β-arrestin mediated endocytosis has been shown to lead to the activation of signaling events, including those in the MAPK pathway. MAPK signaling can then lead to the activation of another group of signaling proteins, extracellular–signal regulated kinases (ERK). The Maginnis Laboratory has previously demonstrated that ERK is crucial to JCPyV infection (Dushane et al., submitted manuscript). Because β-arrestin has been implicated as an activator of ERK in other studies, this further suggests that β-arrestin aids in JCPyV entry in a clathrin-mediated entry process and the activation of
scaffolding proteins at the time of entry may be impacting activation of downstream signaling cascades crucial for the JCPyV infectious lifecycle.

The data presented in this thesis demonstrates the entry mechanism utilized by JCPyV in SVG-A cells. Based on the reported findings, we have generated a working model for JCPyV entry (Fig. 12). Upon viral activation of the 5-HT$_2$R, β-arrestin binds to the receptor, likely causing the uncoupling of the G-proteins, which leads to receptor desensitization and subsequent internalization. β-arrestin causes further recruitment of scaffolding proteins AP2, and AP180, which lead to the recruitment of clathrin. AP2 and AP180 have been reported to act in cooperation to recruit and stabilize clathrin at the plasma membrane. Collectively, the presence of these proteins within the cell are crucial to JCPyV entry into permissive host cells.

In recent years, the incidence of PML has increased in the HIV/AIDS population and those undergoing immunosuppression therapies for immunological disorders, such as multiple sclerosis (8). If left untreated, PML is a rapidly fatal, devastating disease. Treatment options for PML are currently ineffective and inadequate, and sometimes lead to the patient’s condition worsening (7,8). In order to develop effective therapies, the scientific community must first understand the mechanisms of JCPyV entry, as it
illuminates targets for potential antiviral therapeutics. The present study provides new information confirming that JCPyV enters through clathrin-mediated endocytosis and that key scaffolding proteins for receptor internalization, β-arrestin, AP2, AP180, and clathrin, are also crucial to JCPyV entry.

These proteins can serve as new targets for therapeutics to prevent PML. Approximately 25% to 30% of drugs on the market utilize GPCRs as a target (35). The mechanism of many of these treatments, including the SSRI drugs and GPCR-targeting drugs that have been utilized for treatment in PML, is still unknown. The present research contributes to efforts to produce efficacious drugs by determining that β-arrestin, AP2, AP180, and clathrin are crucial to JCPyV entry, thus providing new targets for potential treatments. Indeed, some of the β-blocker class of GPCR-targeting drugs on the market, such as Nebivolol, have been shown to specifically target β-arrestin (39). Drugs like these give us confidence that understanding the biological basis of viral entry, specifically the role of scaffolding proteins like β-arrestin, can optimize treatment efficacy. Thus, if we are able to contribute to the design of a new drug that has the potential to limit JCPyV entry and subsequent spread of the virus, the incidence of PML may be reduced. For example, a β-arrestin- or 5-HT2R-specific drug could be combined with mefloquine, an anti-malarial drug that has demonstrated decreased JCPyV infection at a post-entry step (24), in a dual therapy paradigm to limit the progression or development of PML. This treatment option could not only lead to cognitive improvements in patients with PML, but also ultimately reduce the number of cases of PML that develop; it has been demonstrated that dual treatments of mefloquine with a
serotonin antagonist have, in some cases, reduced PML symptoms and JCPyV presence in the CSF (39).

Collectively, this data has provided new information about the relationship between virus-to-host interactions during viral entry. This research contributes not only to the understanding of the fatal JCPyV infection, but it can also be applied to a broader understanding of viral pathogenesis and its implications in human disease, ultimately leading to the development of effective therapeutics against opportunistic viral pathogens.
References


Author Biography

Conner “CJ” Lajoie is originally from Yarmouth, ME where he graduated from Yarmouth High School in 2013. Conner began studying at The University of Maine Honors College in the fall of 2013. He did his first research internship at the Mount Desert Island Biological Laboratory through the INBRE Comparative Functional Genomics Summer Research Fellowship. He spent one summer working at the Jackson Laboratory for Genomic Medicine under the direction of Dr. Edison Liu and Dr. Francesca Menghi. Finally, he spent two academic years under the mentorship of Dr. Melissa Maginnis, which is when his passion for research was ignited. While an undergraduate, he presented his work at six research symposia. Conner has received several grants and fellowships, including the INBRE Functional Genomics Junior and Thesis Fellowships, and is honored to receive the Professor Frederick H. Radke Award from the Department of Molecular and Biomedical Sciences. Conner is graduating with highest honors from The University of Maine Honors College.

After graduation, Conner plans to work for the Broad Institute of MIT and Harvard for a couple of years before attending medical school. When not in the laboratory, Conner spends his time hiking, swimming, or doing any other outdoor activity.